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Optimization of the conventional minimum inhibitory concentration method for drug susceptibility testing of ethionamide

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ABSTRACT

Evaluation of newer methods and optimization of existing methods for the susceptibility testing of second-line drugs, especially ethionamide, are essential when treatment of multidrug-resistant tuberculosis (MDR-TB) is warranted. The ideal method must clearly demarcate sensitive from resistant strains. Hence, optimization of the conventional minimum inhibitory concentration (MIC) method was attempted using diluted inoculum. The optimized MIC method was evaluated using 206 *Mycobacterium tuberculosis* strains isolated from new and previously treated tuberculosis patients and were compared with the conventional MIC method and proportion sensitivity (PST) method. The sensitivity and specificity of the optimized MIC method in comparison with the PST method was 74% and 90%. Assessment of the optimized MIC method with the conventional MIC method gave a sensitivity of and specificity of 73% and 98%. Overall agreement between the zmethods was found to be ⩾80%. Endowed with the ability to identify the resistant strains precisely, the optimized MIC method can be used for screening resistance to ethionamide.

Introduction

Emergence of multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB) is of great epidemiological concern, especially in tuberculosis (TB) endemic settings like India [1]. The estimated proportion of MDR-TB worldwide is 2.9% and 15.3% from new and previously treated cases [2]. Ethionamide (ETO) is used in programmatic management of drug-resistant tuberculosis (PMDT) under the Revised National TB Control Programme (RNTCP) for the treatment of multidrug resistant TB (MDR-TB) [3]. Therefore, determination of a susceptibility profile for ETO is warranted for effective monitoring and customized treatment. An accurate method for drug susceptibility testing (DST) of this thermo labile drug has been difficult to establish because the difference in minimum inhibitory concentration (MIC) associated with resistance is minimum. Hence, the distribution of probable sensitive and resistant strains is not well separated [4,5] leading to a discrepancy between MIC values of ETO and the clinical outcome of patients. Efforts are required to revisit the MIC values at regular intervals to ascertain the breakpoint concentration. Another approach is to validate

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new methodologies or optimize existing methods for the same. Herein the optimization of the existing conventional MIC method for ethionamide by using diluted inoculum (1 μg/ml) is reported.

Materials and methods

A total of 235 *M. tuberculosis* strains isolated from new and previously treated TB patients were subjected to DST for ETO by MIC and PST methods following standard procedure [6,7]. Briefly, one-third loopful of 2–3 week-old culture on Lowenstein–Jensen (LJ) media was suspended in 1 ml of sterile distilled water and vortexed to obtain an even suspension of 4 mg/ml. The coarse particles or clumps in the suspension were allowed to settle at room temperature; 10 microliters of the suspension was inoculated onto drug and drug-free LJ medium. The concentration of the ETO used was 20, 28.5, 40, 57, 80, 114 and 156 μg/ml. The inoculum concentration used for conventional MIC (4 mg/ml) was diluted using sterile distilled water to obtain diluted inoculum of 1 mg/ml concentration. Susceptibility testing for ETO using diluted inoculum at the above-mentioned drug concentrations was performed by the conventional MIC method described above. Tenfold dilution from 1 mg/ml concentration was prepared by adding 0.2–1.8 ml sterile distilled water (S1, 10^{-1}). Two further serial dilutions 10^{-2} (S2) and 10^{-3} (S3) were prepared in a similar manner. Ten microliters from each of the above dilutions were inoculated onto drug-free and drug-containing LJ slopes (40 μg/ml of ethionamide). Susceptibility testing was carried out at the same time point using the same batch media to avoid any error. Results were read after 28 days and 42 days of incubation at 37 °C for MIC and PST methods respectively.

Interpretation of conventional and optimized MIC methods

Isolates with ⩾20 colony counts (1+ grading) were considered resistant to the particular drug concentration of ETO. The colony count for determining resistance was kept equal for both methods. Break point MIC value for defining resistance in the conventional MIC method was ⩾80 μg/ml and values less than that were considered susceptible.

Interpretation of PST method

Isolates with more than 1% of colony forming units (CFU) in drug-containing LJ slopes in comparison with drug-free LJ slopes were considered as resistant by the PST method. Isolates with values less than 1% criteria defining resistance were considered as susceptible. Isolates with PST values between 0.9% and 1.1% were considered as "borderline".

Errors

The presence of false resistance or susceptibility is considered as errors in any method [8]. False resistance (FR) is classified as major error (ME), which does not have any major implications with respect to treatment of patients. But false susceptibility (FS) is considered to be very major error (VME) as it guides improper treatment for the patient.

Laboratory susceptible strain $H_{37}Rv$ was used as a control on a daily basis. The results were analyzed by Chi Square test using SPSS software version 17.0.

Results

Two hundred and six isolates out of 235 *M. tuberculosis* strains had valid results in DST for ETO by conventional MIC, optimized MIC and PST methods. The pattern of susceptibility obtained by the optimized MIC method showed a varied distribution of isolates along the drug concentrations used (Table 1). The presence of isolates with resistance more than the highest concentration (114 μg/ml) routinely used in DST for ETO was observed. The standard strain $H_{37}Rv$ showed a susceptible result during the DST time line.

It was re-determined that 80ug/ml was the most appropriate breakpoint minimum inhibitory concentration for the conventional MIC method (communication under process). Since the optimized MIC method uses diluted inoculum, use of the same breakpoint concentration (80 μg/ml) needs to be assessed. The susceptibility pattern for the optimized MIC method with breakpoint MIC values at 80, 114 and 156 μg/ml were analyzed and compared with the PST and the conventional MIC methods.

Evaluation of the optimized MIC method with the PST method

The optimized MIC method was compared with the "standard" PST method for 206 isolates; 4 isolates that showed borderline PST values were eliminated. Agreement between the optimized MIC method at 80 μg/ml and the PST method was found to be superior (kappa value – 0.590) to 114 and 156 μg/ml (Table 2). An increase in the number of VME was observed with an increase in breakpoint concentration from 80 to 114 and 156 μg/ml. As expected, the ME was found to decrease with an increase in the breakpoint concentration. Although slightly improved specificity was observed with increasing breakpoint concentrations, by overall comparison with the PST method, 80 μg/ml was found to be the "effective" breakpoint concentration for the optimized MIC method.

Table 1 – Pattern of MIC values obtained for ethionamide by optimized MIC method.

	Minimum inhibitory concentration (in μg/ml)								Total
	20	28.5	40	57	80	114	156	>156	
No. of strains	27	10	31	34	24	31	20	29	206

Table 2 – Evaluation of Optimized MIC method with PST method (at 40 μg/ml).

Optimized MIC value	Susceptibility profile	PST method at 40 μg/ml		Total
		Resistant	Susceptible	
⩾80 μg/ml	Resistant	97	7	104
⩽80 μg/ml	Susceptible	34	64	98
	Total	131	71	202
Sens: 74%; Spec: 90%; PPR: 93%; PPS: 65%; Accuracy: 80%; kappa: 0.590 (moderate); CI(95%): 0.483–0.698				
⩾114 μg/ml	Resistant	75	5	80
⩽114 μg/ml	Susceptible	56	66	122
	Total	131	71	202
Sens: 57%; Spec: 93%; PPR: 94%; PPS: 54%; Accuracy: 68%; kappa: 0.431 (moderate); CI(95%): 0.326–0.537				
⩾156 μg/ml	Resistant	48	1	49
⩽156 μg/ml	Susceptible	83	70	153
	Total	131	71	202
Sens: 37%; Spec: 99%; PPR: 98%; PPS: 46%; Accuracy: 57%, kappa: 0.279 (fair); CI(95%): 0.194–0.363				

MIC value: the MIC value obtained equal, less or more than the specified concentration; PST – proportion sensitivity testing; Sens – sensitivity; Spec – Specificity; PPR/PPS – positive predictive value for resistance/susceptible; CI – confidence interval at 95%.

Evaluation of the optimized MIC method with the conventional MIC method

The minimum inhibitory concentration of 206 isolates obtained by the conventional MIC method and the optimized MIC method was compared. When the breakpoint MIC was arbitrarily set at 80 μg/ml, a single FR result was noted by the optimized MIC method (Table 3). However, various breakpoint concentrations were analyzed for the optimized MIC method in comparison with the conventional MIC method at 80 μg/ml (Table 4). The predictive value for resistance was higher when the MIC was set at 80 μg/ml than at the other two concentrations. When the MIC for the optimized MIC method was set at 80 μg/ml, a single FR and 39 FS were observed with a kappa value of 0.610. Though the statistical values at MIC (156 μg/ml) were higher, the ME and the VME were more than that observed at 80 μg/ml. Results of MIC at 114 μg/ml indicated more or less an even distribution of ME and VME.

Discussion

Ethionamide is used in the treatment of MDR-TB under RNTCP [9]. Therefore, it becomes necessary to verify or, if required, re-standardize the susceptibility testing method for the drug. Effective drug action and discrimination of resistant and susceptible isolates can be observed whenever the MIC of the drug is well below its therapeutic index [10]. The MIC of ETO is very close to its therapeutic index especially in solid LJ medium, thus increasing the chance of additional resistance [5]. It is known that some variation in MIC for ETO is expected in different laboratories and also with respect to time intervals [6] which can be attributed to a

Table 3 – Comparison of MIC values obtained by conventional and optimized MIC methods.

Optimized MIC method (in μg/ml)	Conventional MIC method (in μg/ml)								Total
	⩽20	28.5	40	57	80	114	156	>156	
⩽20	8	9	5	5	0	0	0	0	27
28.5	1	1	2	5	0	1	0	0	10
40	0	1	4	14	11	1	0	0	31
57	0	1	2	5	12	11	3	0	34
80	0	0	0	0	7	9	5	3	24
114	0	0	0	0	0	5	15	11	31
156	0	0	0	1	0	1	13	5	20
>156	0	0	0	0	0	1	1	27	29
Total	9	12	13	30	30	29	37	46	206

MIC value: the MIC value obtained equal, less or more than the specified concentration.

Table 4 – Evaluation of MIC values between conventional and optimized MIC methods.

Optimized MIC value	Susceptibility profile	Conventional MIC method at 80 μg/ml		Total
		Resistant	Susceptible	
⩾80 μg/ml	Resistant	103	1	104
⩽80 μg/ml	Susceptible	39	63	102
	Total	142	64	206
Sens: 73%; Spec: 98%; PPR:99%; PPS: 62%; Accuracy: 81%; kappa: 0.610 (good); CI(95%): 0.509–0.711				
⩾114 μg/ml	Resistant	50	30	80
⩽114 μg/ml	Susceptible	25	101	126
	Total	75	131	206
Sens: 67%; Spec: 77%; PPR: 63%; PPS: 80%; Accuracy: 73%; kappa: 0.432 (moderate); CI(95%): 0.305–0.558				
⩾156 μg/ml	Resistant	32	17	49
⩽156 μg/ml	Susceptible	14	143	157
	Total	46	160	206
Sens: 70%; Spec: 89%; PPR: 65%; PPS: 91%; Accuracy: 85%; kappa: 0.576 (moderate); CI(95%): 0.443–0.709				

MIC value: the MIC value obtained equal, less or more than the specified concentration; Sens – sensitivity; Spec – specificity; PPR/PPS – positive predictive value for resistance/susceptible; CI – confidence interval at 95%.

variation in the level of "local" strain types of *M. tuberculosis* circulating within the geographical region. Such differences may contribute to the disparity between *in vivo* and *in vitro* drug susceptibility profiles, thus hampering effective treatment [11].

Considering the above facts, this study attempted to revisit the existing methodology with a novel approach by diluting the inoculum used for the conventional MIC methodology. The assumption was whether enhanced discrimination between resistant and susceptible isolates can be achieved by diluting the inoculum rather than using a concentrated inoculum of 4 mg/ml. As expected, a decrease in the culture grading in drug-free as well as in drug-containing slopes was observed using a diluted inoculum compared to that of the conventional method, which was consistent (data not shown). A wide distribution of isolates with different MIC values is an indicator of robustness and applicability of the conventional MIC method even if modifications are introduced.

A slight decrease in the sensitivity of the optimized format in comparison with the conventional MIC (73%) and the PST (74%) methods can be attributed to the presence of false susceptible isolates. Out of 73 VME isolates from both conventional MIC and PST methods, 65 isolates had their MIC values distributed at 57 and 40 μg/ml. Isolates with greater deviation in MIC either on the higher or lower scale was limited. Reasons for the VME may be the dilution effect on the inoculum. There exists a possibility to miss the resistant population if present in fewer quantities. Use of a higher inoculum might sometimes indicate a "high" rate of resistant phenotype that may not be a true resistant at all times and interference of clumps becomes inevitable [12].

Seven isolates were classified as ME by the optimized MIC method when compared with the PST method. Four of these had an MIC value of 114 μg/ml, a single isolate had a value of 156 μg/ml and the remaining two isolates had an MIC of 80 μg/ml. The reason for the presence of ME may be attributed to a technical defect, especially during the inoculation process. A nichrome wire loop with a 3 mm diameter that is able to deliver 10 μl is used for inoculation onto LJ slopes. Though the method is standardized and has been used for decades in this laboratory, sometimes an error occurs when the inoculum size is increased and such fault can be rectified.

Errors (ME and VME) could also be due to the presence of "borderline" isolates in both the methods. Such isolates tend to indicate varying susceptibility patterns at each episode. Molecular analysis of these ME and VME isolates might explain the reasons for a discrepancy, if any, and involvement of single or multiple genes associated with resistance. The involvement of more than one gene conferring resistance, as in drugs like streptomycin and isoniazid, could be one of the reasons for the presence of "borderline" resistant isolates. The exact mechanism of resistance for ETO has not yet been fully deciphered, but the inhibition of fatty acid synthesis was identified as the drug target in *M. tuberculosis* [13–15]. It is also speculated that resistance to ETO might be mediated through multiple genes and thus can have "borderline" resistant isolates [11,16–17].

It is expected that an ideal method should provide results similar or better than the other methods available. However, it should be noted that variations arising due to differences in the methodology still remains and they play a vital role in determining the accuracy of the method. Such variations lead to the presence of errors. The minimum inhibitory concentration method determines or measures the level of the drug that can be used for effective treatment whereas PST measures the population of the bacteria that overcome the effect of the drug. There exists a variation in the basis between these methods defining resistance [5].

Varying inoculum size was not compared with the conventional method because the optimized format is the initial dilution (S1 dilution according to RNTCP manual [18]) used in the proportion sensitivity testing (PST) method, which is a qualitative DST method. When the concentration of inoculum is still reduced (1 in 10 dilution of optimized format), colony counts will further decrease. In such cases, an effective comparison could be obtained and results in these inoculum concentrations may not depict "true" susceptibility to the MIC method.

The optimized methodology is validated using different patient populations comprising new patients, patients

treated with first-line drug therapy, and patients on treatment for MDR-TB. With overall high accuracy, the optimized method can also be used as an alternative to the conventional MIC method to determine the susceptibility profile of ETO. The optimized method can be used in resource-limited settings where DST is still performed on LJ medium. There is no cost reduction by using this optimized format, but it could provide a better demarcation between resistant and susceptible isolates. The proportion sensitivity testing method is being performed for patients enrolled for treatment of MDR-TB. The use of an optimized format might prove to be a better alternative for the existing methodology and results can be obtained 2 weeks earlier than the PST method. Any strain identified as resistant by the optimized method can be considered as such without any further confirmation. However, susceptible isolates need to be reconfirmed, preferably using liquid culture systems like MGIT 960.

Conclusion

The present study is a novel attempt to optimize the widely used MIC method for susceptibility testing of ethionamide, one of the second-line drugs used in MDR-TB treatment in the country. For drugs that confer uncertain DST results, there arises a need for a method that can effectively discriminate the susceptible and resistant isolates. The study indicates that the optimized format of the conventional MIC method can be used for susceptibility testing. However, stringent dilution might enhance the efficiency of the method.

Conflict of interest

No conflict of interest is perceived and none declared.

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